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SERIAL NO: 09/997,209	FILING DATE: 11/28/01	EXAMINER: T. Wessendorf	GROUP ART UNIT: 1639 CONFIRMATION NO.: 3759
INVENTION: EUKARYOTIC EXPRESSION LIBRARIES AND METHODS OF USE			

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Transmitted herewith are the following documents in connection with the above-identified application:

1. Request for Corrected Patent Application Publication (in duplicate)

2. A marked version of the publication showing the corrections

3. A copy of Table 1 and portion of Table 3 showing the correct alignment.

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Respectfully submitted,

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PATENT
Client-Matter No.: 66797-132
(P-IX 5066)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: William D. Huse	Group Art Unit: 1639
Serial No.: 09/997,209	Examiner: T. Wessendorf
Filed: November 28, 2001	Confirmation No.: 3759
For: EUKARYOTIC EXPRESSION LIBRARIES AND METHODS OF USE	

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Mail Stop PGPUB
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

REQUEST FOR CORRECTED PATENT APPLICATION PUBLICATION

The Applicants respectfully request a corrected patent application publication under 37 C.F.R. § 1.221(b).

The Applicants believe that publication No. US-2003-0096401-A1, published May 22, 2003, contains the following material mistakes that are apparent from USPTO records:

On page 18, the numbers on row "CDRL1" should be on row "Amino Acid".

On page 19, below header please delete "Amino Acid".

On Page 19, Table 1, under CDR L1, row labeled "M131B-11", second to last column above "Met," please delete "ATO" and substitute therefor with **"ATG"**.

On Page 19, Table 1, under CDR L1, row labeled "M131B3-12" the nucleic and amino acid sequence is misaligned. The nucleotide and amino acid sequence of row "M131B3-12" should read:

AGC CAG AGT GCT AAG CAT ATG AAC

Ser Gln Ser Ala Lys His Met Asn

On page 23, Table 3, Library 1; under "Sequence" row labeled "1.8"; please delete "D" and substitute therefor with **"G"**.

On Page 23, Table 3, Library 2; under "Sequence" row labeled "2.6"; "I" is out of position. Please move "I" one space to the left, aligned under the "N."

For convenience, attached is a marked version of the publication showing the corrections. Also attached is a copy of Table 1 and portion of Table 3 showing the correct alignment.

Accordingly, Applicants request that these errors be corrected in the USPTO's electronic copy of the Specification and that the USPTO publish a corrected patent application publication.

No fee is deemed necessary to file this Request. If any fee is required, authorization is hereby given to charge the

amount to Deposit Account No. 502624. A duplicate copy of this sheet is enclosed for this purpose.

Respectfully submitted,

July 22, 2003
Date

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[0163] These results show that ligands and receptors can be represented as vectors to determine the probability of identifying a ligand that binds to a receptor.

EXAMPLE V

Optimization of Anti-idiotypic Antibody Ligands

[0164] This example shows that screening ligands with receptor variants increases the probability of identifying an optimal binding ligand.

[0165] The parent receptor was antibody BR96, a mouse monoclonal antibody to Le^Y-related cell surface antigens. Six receptor variants were generated using random codon synthesis as described in U.S. Pat. No. 5,264,563 and in Glaser et al. supra. Briefly, synthesis was performed using two DNA synthesizer columns. For simplicity, the DNA sequences are referred to as the coding strand although, in practice, all oligonucleotides were synthesized as the complementary sequence. On column 1 a trinucleotide coding for the predetermined parental codon found at the CDR positions specified below was synthesized. On column 2 a random codon encoding all 20 amino acids was synthesized using the nucleotides XXG/T where X represents a mixture of dA, dG, dC and T cyanoethyl phosphoramidites. The use of the XXG/T codon reduces the number of stop codons to include only UAG, which can be suppressed in supE *E. coli* bacterial strains. After synthesis of each codon, the beads from the two columns were mixed together, divided in half, and then repacked into two new columns. The columns were then returned to the DNA synthesizer and the process was repeated for the subsequent CDR positions. After the final synthesis step the contents of the two columns were pooled and the resulting oligonucleotides purified. This particular application of codon-based synthesis results in a mixture of oligonucleotides coding for randomized amino acids within a predefined region while maintaining a 50% bias toward the parental sequence at any position. By altering the proportion of the beads in the two columns, the level of substitution with respect to parental sequence can be further controlled. Furthermore, any given position can retain a specified codon and mixtures of codons other than XXG/T can be used to insert only some subset of amino acid residues if desired.

[0166] Oligonucleotides containing randomized codons were used to generate receptor variants by mutagenesis (Kunkel, *Proc. Natl. Acad. Sci. USA* 82:488-492 (1985) and Kunkel et al., *Methods Enzymol.* 154:367-382 (1987)). Briefly, M13IXL604 or M13IXL605 phage were grown in the dut$^-$ ung$^-$ *Escherichia coli* strain CJ236 (BioRad, Richmond, Calif.) and phage were precipitated by adding 0.25 volumes of 3.5 M ammonium acetate, 20% polyethylene glycol/ml of cleared culture supernatant. Uracil-substituted single stranded DNA was isolated by phenol extraction followed by ethanol precipitation. From 6 to 8 pmol of phosphorylated oligonucleotide were used to mutagenize 250 ng of the chimeric L6 template in a 13 μl reaction volume (Huse et al., *J. Immunol.* 149:3914-3920 (1992). The reaction products were diluted twofold with water and 1 μl was electroporated into *E. coli* strain XL-1 (Stratagene, San Diego, Calif.) and titered onto a lawn of XL-1.

[0167] Three anti-idiotypic antibody ligands were generated by immunizing 6 or 7-week-old BALB/c mice intraperitoneal (four times, once every 20 days) with 50 μg of purified antibody BR96 using aluminum hydroxide as adjuvant. The reactivity of the mice sera was tested by ELISA (Fields et al., *Nature* 374:739-742 (1995)). After a final boost with soluble polyclonal rabbit IgG, mice with the strongest response were killed and the spleens were used to obtain hybridomas as described (Galfre and Milstein, *Methods Enzymol.* 73:3-46 (1981)).

[0168] Receptor variants were screened for binding to anti-idiotypic antibody ligands. The anti-idiotypic antibody ligands were screened against the parent receptor and six receptor variants to determine binding activity using an ELISA assay (see FIG. 3). Anti-idiotypic antibody No. 1 was classified as binding to receptor 12 and the parent receptor. Anti-idiotypic antibody No. 7 was classified as binding to receptor 7, receptor 10 and the parent receptor. Anti-idiotypic antibody No. 3 was classified as binding to all of the receptors, including the parent receptor.

[0169] The nucleotide and amino acid sequences of the light chain CDR regions 1 and 2 of the parent receptor (designated wild type) and the six receptor variants (designated M13IB3-5 through M13IB3-12) are shown in Table 1. The nucleotide and amino acid sequences (SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, and 2, 4, 6, 8, 10, 12, 14, respectively) for the CDR L1 region of the parent and six receptor variants are shown in the top half of Table 1. The nucleotide and amino acid sequence (SEQ ID NOS: 15, 17, 19, 21, 23, 25, 27 and 16, 18, 20, 22, 24, 26, 28, respectively) for the CDR L2 region of the parent and six receptor variants are shown in the bottom half of Table 1. In Table 1, L1 and L2 CDR mutations in M13IXL604 clones were selected on the basis of binding to anti-idiotypic antibody No. 3 similar to that of wild type and negligible binding to anti-idiotypic antibody No. 1. Changes resulting from the mutagenesis procedure are indicated by boldface type.

[0170] Several positions in the receptor sequence were found to be conserved while other positions were found to differ from the parent receptor in both CDR regions 1 and 2. Substitutions occurred at all five target loci in CDR L1 and at three loci in CDR L2. The total number of substitutions in CDR L1 and CDR L2 ranged from two to four in each mutant.

TABLE 1

Nucleotide and Amino Acid Sequences of Receptor Variants of BR96 Antibody

Amino Acid								
CDR L1	26	27	28	29	30	31	32	33
Wild type	AGC	TCA	AGT	GTA	AGT	TTC	ATG	AAC
	Ser	Ser	Ser	Val	Ser	Phe	Met	Asn

TABLE 1-continued

Nuclectide and Amino Acid Sequences of Receptor Variants of BR96 Antibody

M131B3-5	AGC	TCA	AGT	GTA	AGG	TTC	ATG	AAC
	Ser	Ser	Ser	Val	Arg	Phe	Met	Asn
M131B3-6	AGC	GAG	AGT	GTA	AAT	CTT	ATG	AAC
	Ser	Glu	Ser	Val	Asn	Leu	Met	Asn
M131B3-7	AGC	TCA	AGT	GTT	AAT	TTC	ATG	AAC
	Ser	Ser	Ser	Val	Asn	Phe	Met	Asn
M131B3-10	AGC	TCA	ACG	GTA	AGT	TTC	ATG	AAC
	Ser	Ser	Thr	Val	Ser	Phe	Met	Asn
M131B3-11	AGC	TCA	AGT	GTA	GCG	TAT	ATG	AAC
	Ser	Ser	Ser	Val	Ala	Tyr	Met	Asn
M131B3-12	AGC	CAG	AGT	GCT	CAT	ATG	AAC	
	Ser	Gln	Ser	Ala	AAG Lys	His	Met	Asn
CDR L2	49	50	51	52	53	54	55	56
Wild type	GCC	ACA	TCC	AAT	TTG	GCT	TCT	GGA
	Ala	Thr	Ser	Asn	Leu	Ala	Ser	Gly
M131B3-5	GCC	ACA	GAG	AAG	TTG	GCT	TCT	GGA
	Ala	Thr	Glu	Lys	Leu	Ala	Ser	Gly
M131B3-6	GCC	ACA	GTT	AAT	TTG	GCT	TCT	GGA
	Ala	Thr	Val	Asn	Leu	Ala	Ser	Gly
M131B3-7	GCC	ACA	GTG	AAT	TTG	GCT	TCT	GGA
	Ala	Thr	Val	Asn	Leu	Ala	Ser	Gly
M131B3-10	GCC	ACA	TCC	AGG	GCG	GCT	TCT	GGA
	Ala	Thr	Ser	Arg	Ala	Ala	Ser	Gly
M131B3-11	GCC	ACA	CAG	AAT	TTG	GCT	TCT	GGA
	Ala	Thr	Gln	Asn	Leu	Ala	Ser	Gly
M131B3-12	GCC	ACA	TCC	AAT	TTG	GCT	TCT	GGA
	Ala	Thr	Ser	Asn	Leu	Ala	Ser	Gly

[0171] The results of the screen are summarized in FIG. 6, where receptors are represented as discs and ligands are represented as symbols. These results demonstrate that screening ligands against a population of receptor variants will rapidly identify ligands having optimal binding activity. For example, if the collective receptor variant population of this example were screened in the melanophore system, ligand No. 3 would have generated the highest signal since it binds to all seven receptors in the receptor variant population. Ligand No. 7 would give a weaker signal since this ligand binds to three receptors in the receptor variant population. Ligand No. 1 would give a still weaker signal since this ligand binds to two receptors in the receptor variant population. Thus, screening with a collective receptor variant population provides more information about the binding characteristics of the ligand than screening with the parent receptor alone. In addition, ligands that bind weakly to the parent receptor may not have been detectable above background when screened against the parent alone but are detectable when more than one receptor in the receptor variant population binds to the ligand.

[0172] These results demonstrate that screening a receptor variant population rapidly identifies optimal binding ligands to a receptor.

EXAMPLE VI

Modification of the Doublelox Targeting Vector

[0173] This example describes modification of the doublelox targeting vector.

[0174] The doublelox targeting vector pBS397-p53cat could not be used as a general vehicle for applying directed evolution technologies to a wide range of proteins because the synthetic polylinker region contained a limited number of unique restriction sites that hindered rapid cloning of the target protein(s) of interest. Moreover, the vector did not contain the filamentous phage origin of replication and, consequently, could not be used to generate single-stranded

TABLE 3-continued

Summary of amino acid sequence of randomly selected BRP variants.

Library	# Sequenced	Designation	n	Sequence
		1.6	1	Y
		1.7	1	L
		1.8	1	G
		1.9	1	S
		1.10	1	R
		1.11	1	(deletion)
2	18	WT	5	V T L F I S A V Q D
		2.1	2	L
		2.2	1	A
		2.3	1	L
		2.4	1	V
		2.5	1	N
		2.6	1	I
		2.7	1	T
		2.8	2	H
		2.9	1	P
		2.10-11	1	(silent mutations)
3	18	WT	7	D N T L A W V W V
		3.1	2	D G
		3.2	1	L G
		3.3	1	P G
		3.4	1	M G
		3.5	1	C
		3.6	1	S
		3.7	1	G W
		3.8	1	G R
		3.9	1	G L
		3.10	1	C
4	18	WT	9	T S I G E Q P W G R S F A
		4.1	1	V
		4.2	1	S
		4.3	1	W
		4.4	2	H
		4.5	1	L
		4.6	1	G

TABLE 3-continued

Summary of amino acid sequence of randomly selected BRP variants.

Library	# Sequenced	Designation	n	Sequence
		4.7	1	S
		4.8-9	1	(silent mutations)

[0202] These results describe the generation of focused BRP libraries. Hybridization mutagenesis of BRP using oligonucleotides synthesized by codon-based mutagenesis introduced the desired diversity focused across the regions of interest.

EXAMPLE X

Functional Screening of BRP Libraries Expressed in Mammalian Cells

[0203] This example describes functional screening of BRP libraries expressed in mammalian cells.

[0204] Each of the four BRP libraries was used to transform the mammalian host cell line 13-1 using optimized conditions described in Example VIII, and site-specific integrants were selected with geneticin. Host cells transformed with BRP variants were identified based on resistance to geneticin and subsequently were isolated, expanded, and screened for Zeocin sensitivity (FIG. 7). After proliferation to obtain a sufficient number of cells, each clone was plated in four separate wells to permit exposure to variable concentrations of Zeocin for 14 days. Similar to previous results, clones transformed with wild type BRP were resistant to 500, 1000, and 2500 μg/ml Zeocin but were killed by treatment with 5000 μg/ml Zeocin. Therefore, in order to identify BRP variants with beneficial mutations conferring increased affinity for Zeocin, one sample of all clones was treated with 5000 μg/ml Zeocin. Conversely, to identify mutations that diminished binding to Zeocin, that is, sensitive to 2500 μg/ml Zeocin, cultures of each clone were treated with 500 or 1000 ug/ml Zeocin. Clones that were sensitive to 500 μg/ml Zeocin were not characterized further but presumably include mutations that render BRP nonfunctional due to disruption of critical binding residues or substantial perturbation of the structure of BRP.

[0205] Site-specific targeted integrants were selected by placing the transfected cells in media containing geneticin. Following the outgrowth of colonies, separate cultures of each clone were grown in the presence of the indicated concentration of Zeocin. The phenotypes of the BRP variants were categorized as beneficial (resistant to 5000 μg/ml Zeocin), wild type (resistant to 2500 μg/ml Zeocin), detrimental (resistant to 500 and 1000 μg/ml Zeocin), or nonfunctional (sensitive to 500 μg/ml Zeocin). The variants were categorized as shown in FIG. 7.

[0206] Treatment of the clones transformed with BRP mutants with varying amounts of Zeocin led to the identification of multiple clones displaying altered sensitivities to Zeocin, with detrimental mutations being identified most frequently. The predominance of detrimental mutations foi-

Table 1. Nucleotide and Amino Acid Sequences of Receptor Variants of BR96 Antibody

Amino Acid	26	27	28	29	30	31	32	33
CDR L1								
Wild type	AGC	TCA	AGT	GTA	AGT	TTC	ATG	AAC
	Ser	Ser	Ser	Val	Ser	Phe	Met	Asn
M131B3-5	AGC	TCA	AGT	GTA	**AGG**	TTC	ATG	AAC
	Ser	Ser	Ser	Val	**Arg**	Phe	Met	Asn
M131B3-6	AGC	**GAG**	AGT	GTA	**AAT**	**CTT**	ATG	AAC
	Ser	**Glu**	Ser	Val	**Asn**	**Leu**	Met	Asn
M131B3-7	AGC	TCA	AGT	GTT	**AAT**	TTC	ATG	AAC
	Ser	Ser	Ser	Val	**Asn**	Phe	Met	Asn
M131B3-10	AGC	TCA	**ACG**	GTA	AGT	TTC	ATG	AAC
	Ser	Ser	**Thr**	Val	Ser	Phe	Met	Asn
M131B3-11	AGC	TCA	AGT	GTA	**GCG**	**TAT**	ATG	AAC
	Ser	Ser	Ser	Val	**Ala**	**Tyr**	Met	Asn
M131B3-12	AGC	**CAG**	AGT	**GCT**	**AAG**	**CAT**	ATG	AAC
	Ser	**Gln**	Ser	**Ala**	**Lys**	**His**	Met	Asn

Amino Acid	49	50	51	52	53	54	55	56
CDR L2								
Wild type	GCC	ACA	TCC	AAT	TTG	GCT	TCT	GGA
	Ala	Thr	Ser	Asn	Leu	Ala	Ser	Gly
M131B3-5	GCC	ACA	**GAG**	**AAG**	TTG	GCT	TCT	GGA
	Ala	Thr	**Glu**	**Lys**	Leu	Ala	Ser	Gly
M131B3-6	GCC	ACA	**GTT**	AAT	TTG	GCT	TCT	GGA
	Ala	Thr	**Val**	Asn	Leu	Ala	Ser	Gly
M131B3-7	GCC	ACA	**GTG**	AAT	TTG	GCT	TCT	GGA
	Ala	Thr	**Val**	Asn	Leu	Ala	Ser	Gly
M131B3-10	GCC	ACA	TCC	**AGG**	**GCG**	GCT	TCT	GGA
	Ala	Thr	Ser	**Arg**	**Ala**	Ala	Ser	Gly
M131B3-11	GCC	ACA	**CAG**	AAT	TTG	GCT	TCT	GGA
	Ala	Thr	**Gln**	Asn	Leu	Ala	Ser	Gly
M131B3-12	GCC	ACA	TCC	AAT	TTG	GCT	TCT	GGA
	Ala	Thr	Ser	Asn	Leu	Ala	Ser	Gly

The results of the screen are summarized in Figure 6, where receptors are represented as discs and ligands are represented as symbols. These results demonstrate that screening ligands against a population of receptor variants will rapidly identify ligands having optimal binding activity. For example, if the collective receptor variant population of this example were screened

Table 3. Summary of amino acid sequence of randomly selected BRP variants.

Library	# Sequenced	Designation	n	Sequence
1	16	WT	4	D F V E D D F A
		1.1	1	R
		1.2	1	L
		1.3	1	S
		1.4	1	G
		1.5	2	C
		1.6	1	Y
		1.7	1	L
		1.8	1	G
		1.9	1	S
		1.10	1	R
		1.11	1	(deletion)
2	18	WT	5	V T L F I S A V Q D
		2.1	2	L
		2.2	1	A
		2.3	1	L
		2.4	1	V
		2.5	1	N
		2.6	1	I
		2.7	1	T
		2.8	2	H
		2.9	1	P
		2.10-11	1	(silent mutations)